

NASA-CR-196268

IN-51-012  
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**Rapid Susceptibility Testing of Mycobacterium avium Complex  
and Mycobacterium tuberculosis Isolated from AIDS Patients  
(NASA Research Grant No. NAG10-0106).**

**Final report for the period 01 June 1992 — 31 May 1994**

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N94-36996

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G3/51 0016377

(NASA-CR-196268) RAPID  
SUSCEPTIBILITY TESTING OF  
MYCOBACTERIUM AVIUM COMPLEX AND  
MYCOBACTERIUM TUBERCULOSIS ISOLATED  
FROM AIDS PATIENTS Final Progress  
Report, 1 Jun. 1992 - 31 May 1994  
(Florida Inst. of Tech.) 6 p

In ominous projections issued by both U.S. Public Health Service and the World Health Organization, the epidemic of HIV infection will continue to rise more rapidly worldwide than predicted earlier. The AIDS patients are susceptible to diseases called opportunistic infections of which tuberculosis and M. avium Complex (MAC) infection are most common. This has created an urgent need to uncover new drugs for the treatment of these infections.

In the seventies, NASA scientists at Goddard Space Flight Center, Greenbelt, Maryland, had adopted a biochemical indicator, adenosine triphosphate (ATP), to detect presence of life in extraterrestrial space. We proposed to develop ATP assay technique to determine sensitivity of antibacterial compounds against MAC and M. tuberculosis.

The work was initiated in June 1992. Going through the recent literature, we observed most of the work on ATP was related to bacteria other than mycobacteria. First, there are more than a dozen models of commercially available luminometers; however, all of them use their own reagents, composition of which is not disclosed. So, it was decided to use the Aminco Chem-Glow Photometer for the assay of ATP by bioluminescence technique, since this instrument can be used manually and is very flexible.

#### **REAGENTS:**

Most commercial reagents are provided as a mixture of luciferase & D-luciferin. These gave excessive background light due to impurities in luciferase, and so we decided to prepare our own bioluminescent system.

Luciferase-containing firefly lantern extract (Sigma Chemical Company) was first dissolved in Tris-acetate buffer, pH 7.7 (containing EDTA and 0.5% bovine serum albumin), and passed through G-200 Sephadex column at 4°C. To determine active fractions after elution with the same buffer, 0.2 ml aliquot from each fraction was mixed with 0.1 ml of luciferin solution (400 µg/ml) in the assay cuvette, and to this 0.1 ml of standard ATP solution (100 pg ATP) was injected. Any fraction giving a peak height of

75-100 mm on the recording chart was considered as active fraction. All the active fractions were pooled together and mixed with appropriate amount of luciferin. After gentle mixing, the luciferase-luciferin system was incubated for four hours at 25°C in the dark to permit ATP contaminating in firefly lantern extract to "burn off" to a negligible concentration. The aliquots were then dispensed in amber vials and stored at -20°C. This system reproducibly detected 10 pg ATP/0.1 ml and gave a linear response up to 10,000 pg ATP/0.1 ml. Thus, this system improved the sensitivity by three logs and was 10-15 times less expensive than commercially available preparations. Supplementing this system with additional luciferin did not enhance the sensitivity appreciably.

#### **EXTRACTING AGENTS:**

M. avium strain 9141 (serovar 8) was used for all initial studies. It was grown in Middlebrook 7H9 broth and 7H10 agar plates. The broth suspension was diluted to obtain final concentration of  $1 \times 10^7$  CFU (colony forming units) per ml. For each extraction, 0.1 ml bacterial suspension was centrifuged for 20 minutes at 3,000 rpm; sedimented bacteria were washed twice with 0.05 M Tris buffer, pH 7.75 and finally suspended in the same buffer as desired.

After completing extensive literature search, six extracting agents were selected to extract intracellular ATP from M. avium complex (MAC) and M. tuberculosis (Mtb). These were Tris-EDTA, trichloroacetic acid, Perchloric acid, dimethyl sulfoxide, butanol and chloroform. The optimal extracting procedure was to expose bacteria to chloroform at 100°C for 10 minutes. This method did not inactivate standard ATP, and recovery from bacteria was 94-98%. Also, it was determined that various steps (to be used with sputum and blood samples) such as decontamination with NaOH and removal of mammalian ATP with Triton X-100 had no effect on standard ATP. The ATP content from  $10^6$  cells of M. avium and M. tuberculosis was 544 pg and 572 pg. respectively.

### **Relationship between bacterial counts and ATP levels:**

Using MAC, excellent correlation (coefficient of correlation of 0.946) was obtained between the standard colony count method (7-10 days) and ATP levels (2-3 hours). Next, ATP measurements were made using both MAC and Mtb during the growth cycle. Within 12 hours of incubation (lag phase) the ATP levels showed increase. Again, during logarithmic phase, the increase in colony counts was proportional to increase in ATP levels, while in death phase, decline in ATP levels were more obvious than the colony counts.

### **Effect of antimycobacterial drugs:**

Using common antimycobacterial agents, killing effects of the drug were similar using plate counts and ATP method; however, the minimal inhibitory concentrations (MICs) of these drugs were lower when ATP method was used compared to those obtained with the standard colony count method suggesting higher sensitivity of ATP method. The killing effect of rifampicin on MAC was observed within 24 hours using ATP method, while similar effect was seen four days later using plate count method, again suggesting superiority of ATP method over plate count. Similar observations were also made with Mtb exposed to streptomycin. Drug susceptibility testing was performed on 12 clinical isolates of Mtb using four standard drugs. The results of ATP assay were available after five days of incubation in contrast to three weeks for standard colony count method. The results by both methods agreed well, except that inhibition of M. tuberculosis by ATP method was observed with lower concentration of drugs compared to that observed by colony count method. Nevertheless, same strains were identified as drug-resistant by both the methods.

Next, ATP method was compared with another standard method, BACTEC, using <sup>14</sup>C palmitic acid. MAC and M. tuberculosis were inoculated into 7H9 broth (for ATP assays), 7H11 agar for plate counts and 7H12 broth with <sup>14</sup>C-palmitic acid for BACTEC, each containing various concentrations of antimycobacterial drugs added singly. The

MICs obtained by both ATP and BACTEC methods were the same, but lower than those obtained by plate count method. However, the time to obtain reliable results with ATP method was shorter (3-5 days) than with BACTEC method (5-7 days) and plate count method (1-3 weeks).

The results obtained so far clearly indicate that the ATP method is somewhat superior to colony count method, as well as BACTEC method, in screening potential anti-mycobacterial drugs. However, advantages are (a) ATP method is more economical compared to other two methods, and (b) ATP method does not use any radioactive material, thus avoiding the problem of radioactive waste.

#### **Application to clinical specimens:**

Second part of the project involves application of ATP method to clinical specimens. Development of methods to separate bacteria from sputum specimens took longer time than anticipated initially. Finally, method adopted includes digestion of sputum with sodium lauryl sulfate and NaOH, treating digested material with a detergent, Triton X-100, to rupture all non-bacterial cells followed by treatment with ATPase to inactivate all non-bacterial ATP and finally, extracting bacterial ATP. Several sputum samples from tuberculosis patients were obtained. Again, excellent correlation has been obtained between ATP assay, colony counts and BACTEC method, with ATP assay demonstrating upper edge and having advantages mentioned above.

Next, several sputum specimens were obtained from randomly selected tuberculosis patients. These were untreated in early stages and advanced stages of the disease, as well as partially treated and also who have completed treatment regimens. ATP method (and plate count method) could be applied to specimens from patients in early stages; but BACTEC method could not be applied since organisms recovered from such patients were below the sensitivity limits for BACTEC. Also, ATP method gave useful information on the status of patients under treatment, which could not be obtained by BACTEC method.

Finally, two patients from above series, untreated tuberculosis patients in early stages of the disease, were followed during their entire treatment regimen of six months on a combination antimycobacterial drugs. Within one month of treatment, the ATP levels showed a significant decline suggesting the favorable response of patients to the chemotherapy. Such information could not be obtained either by plate count method or by BACTEC method. However, we could not continue these studies with large patient populations for lack of funds through year three of this grant.

Florida Institute of Technology had agreed in principle to file a patent application for the utility of ATP methods described above. Basically, three patents could have been obtained — one for screening newer anti-mycobacterial compounds against M. tuberculosis and MAC; one for drug susceptibility testing for clinical specimens obtained from tuberculosis and AIDS patients; and third for prognosis of patients under anti-mycobacterial chemotherapy. The patent application was submitted to Research Corporation Technologies for evaluation. According to the report of RCT's Life Sciences Group, "while this may be the first application of this technique to mycobacterial organisms, it is not likely that patent protection adequate for commercial purposes can be obtained. If protection can be obtained for this *in vitro* assay, it is likely to be limited to a method of use. Thus, in order to enforce that protection, it will be necessary to monitor the activities of laboratories and individuals performing the test." Based on this evaluation, Florida Institute of Technology decided not to pursue the patent application.

Even though NASA could not support continuation of this work for the third year, efforts are being made to obtain funds from other sources to complete the most important part of this project — i.e. application of ATP method for rapid follow-up of patients under chemotherapy.